Original Article
Hypoxia-induced up-regulation of Aquaporin-1 in Rat Schwann cells via the MAPK pathway

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Abstract: Background: Aquaporin-1 (AQP1) is a glycoprotein that mediates osmotic water transport. Its expression has been found to be correlated with the swelling of Schwann cells. Hypoxia has been reported to play an important role in inducing the expression of AQP1 during Schwann cell swelling. However, the mechanism that regulates AQP1 expression in Schwann cells remains obscure. Methods: The expression and regulation of AQP1 in Rat Schwann cells was investigated using a tris-gas incubator to build a hypoxia model. Results: The expression of AQP1 was up-regulated upon the induction of hypoxia in cells. Hypoxia also induced the phosphorylation of ERK/p38/JNK MAPK. Specific inhibitors of ERK and p38 MAPK blocked these effects of hypoxia on the expression of AQP1. Conclusion: These findings suggested that AQP1 could be up-regulated in a time-dependent manner by hypoxia at the cellular level and that the regulation of AQP1 in Schwann cells was dependent on ERK and p38 MAPK.

Keywords: Aquaporin-1, Schwann cells, hypoxia, MAPK

Introduction

Aquaporins (AQPs) are a family of channel-forming membrane glycoproteins and are known to mediate cellular water transport, which is driven by a transmembrane osmotic gradient [1]. AQPs, being essential membrane channels, are involved in crucial metabolic processes and are expressed in almost all tissues. Aquaporin-1 (AQP1) has been observed in many cells, including microvascular endothelial beds outside the brain and a variety of tumor cells [2, 3]. The first evidence for the expression of AQPs in the Peripheral nervous system PNS was reported in a study to identify candidate genes involved in somatosensory functions of cranial sensory ganglia [4]. It was revealed that AQPs were involved in many physiological and pathophysiological processes throughout the PNS, including in the trigeminal ganglia [5-8]. However, the functions and regulatory mechanisms of AQP1 in the PNS remain obscure.

In previous study, we had reported that facial nerve tissues expressed AQP1, which was mainly localized in Schwann cells [9-13]. AQP1 up-regulation was shown to be associated with facial nerve edema in the facial palsy model. Some studies have shown that hypoxia could induce AQP1 expression in animal tissue and cell lines [12, 14-17]. Through in vitro cobalt(II) chloride ($CoCl_2$)-induction experiments, we had demonstrated that silencing the expression of AQP1 could inhibit edema [18]. When Schwann cells were exposed to hypoxia, the transcription factor, hypoxia-inducible factor-1 (HIF-1) was activated. Apart from HIF-1, other signaling pathways are also activated by hypoxia, and could potentially regulate AQP1 expression.

The aim of this study was to investigate whether hypoxia could regulate AQP1 expression through MAPKs in Schwann cells and whether specific antagonists of MAPK could inhibit AQP1 expression.

Material and methods

Cell culture

The cell line RSC96 was purchased from Cell Culture Centre, Institute of Basic Medical Science, Chinese Academy of Medical Sciences

(Shanghai, China). The cells were cultured in a DMEM/HIGH GLUCOSE medium supplemented with antibiotics (penicillin and streptomycin; 100 µg/ml) and 10% fetal bovine serum (Gibco, USA) in a humid-atmosphere incubator with 5% CO_2 at 37°C.

Immunofluorescence

The cells were plated on glass coverslips and washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4), before staining. After being fixed with 4% paraformaldehyde for 20 min, the cells were penetrated by 0.5% Triton X-100 at 25°C for 20 min. The nonspecific binding of antibodies was blocked by incubating the cells with PBS containing 10% bovine serum albumin (BSA). The samples were then sequentially incubated overnight at 4°C with primary antibodies against AQP1 (Santa Cruz, 1:200) diluted in a diluent with 1% BSA, and then with fluorescent secondary antibodies, which were FITC-labeled goat anti-mouse IgG antibodies (Beyotime, 1:500). After being washed thrice with PBS for 10 min, the samples were incubated with Hoechst (Sigma, 1:1,000) for 10 min. After being again rinsed with PBS thrice for 10 min, the sections were mounted in glycerol supplemented with an antifade reagent (N-propyl-galat). Microscopic observation was then carried out using a Nikon microscope (Eclipse E600, DXM 1200, Japan). Images were captured using the Nikon ACT-1 software (version 2.63).

Hypoxia-induced Schwann cells

The cells were cultured in a 6-well plate at a density of 1×10^4 cells/cm^2 and maintained in the Tris-gas (2% O_2, 5% CO_2, and 93% N_2, Galaxy) incubator. The expression patterns of AQP1 and the MAPK family signal pathway proteins in these cells were examined at different time points in vitro. To investigate the possible pathway for the induction of AQP1 expression, ERK/P38/JNK specific inhibitor U0126/SB203580/SP600125 (CST) was preincubated for 1 h.

Cell viability tests

Cell viability was determined by ELIASA (Tecan Infinit F50). The cells were cultured in a 96-well plate at a density of 1×10^4 cells/plate and the cell viability was assayed at 0 h, 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h after the induction of hypoxia using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions.

Cell morphological observation

Nine hours after the induction of hypoxia, the morphology of the cells was observed using a digital photocamera (Nikon Coolpix 5000) connected to a phase-contrast microscope (Nikon Eclipse TE200).

Western blot analysis

The cells were cultured at a density of 0.5-1 × 10^5 cells/60 mm cell culture medium. After reaching 70-80% confluence, they were incubated with solutions or inhibitors. hypoxia treatment was performed and the cells were then harvested using the RIPA lysis buffer (Thermo) containing protease inhibitor cocktail (CST) at different time points. These lysates were centrifuged at 12,000 × g for 20 min at 4°C (Thermo). The supernatant was collected and stored at -80°C. The samples (30 µg) were separated using 10% SDS PAGE. After completing the electrophoresis, the proteins were transferred onto PVDF membranes. Non-specific binding sites were blocked by pre-incubating the membranes in 5% non-fat milk in a Tris-buffered saline Tween (TBS-T, 20 mmol/L Tris, 137 mmol/L NaCl, pH 7.6). The PVDF membranes were then incubated overnight at 4°C with specific antibodies: anti-ERK1/2 (CST, 1:2000), anti-p-ERK, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK (CST, 1:1000), anti-AQP1 (Santa Cruz, 1:200), and anti-GAPDH (CST, 1:1000). They were then washed thrice with the TBS-T buffer and incubated with the secondary antibody: alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (CST, 1:2000). Peroxidase labeling was visualized using enhanced chemiluminescence labeling with an ECL Western blot detection system (Thermo). The Western blot bands were scanned and their intensities were analyzed using Image-J for quantification.

Statistical analysis

Data were expressed as the mean ± SD. The significance of difference was evaluated with one-way ANOVA followed by the Student-Newman-Keuls test. Different levels of significance were decided based on the P values: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).
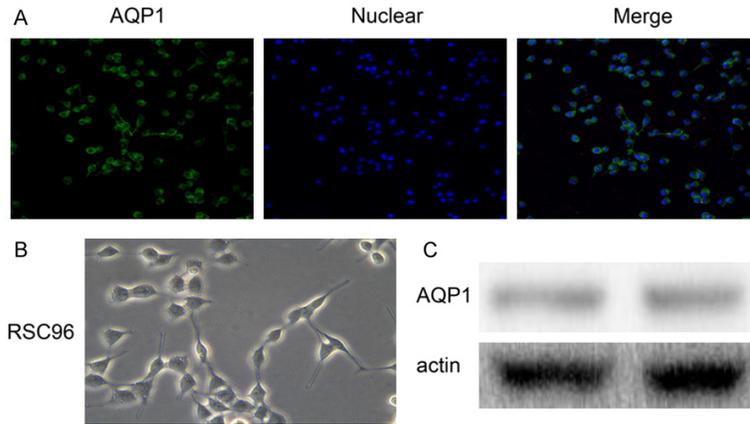
Figure 1. A. Immunostaining images of AQP1 expression in Schwann cells with anti-AQP1 antibody. AQP1 expression (green), Hoechst labeling of the nucleus (blue), Overlay image of AQP1 and Hoechst (Merge). B. Fusiform and long bipolar morphology cells in cultures expresses of the cell line RSC96. C. Western blot analysis of AQP1 protein expression in Schwann cells, actin was used as for protein loading control.
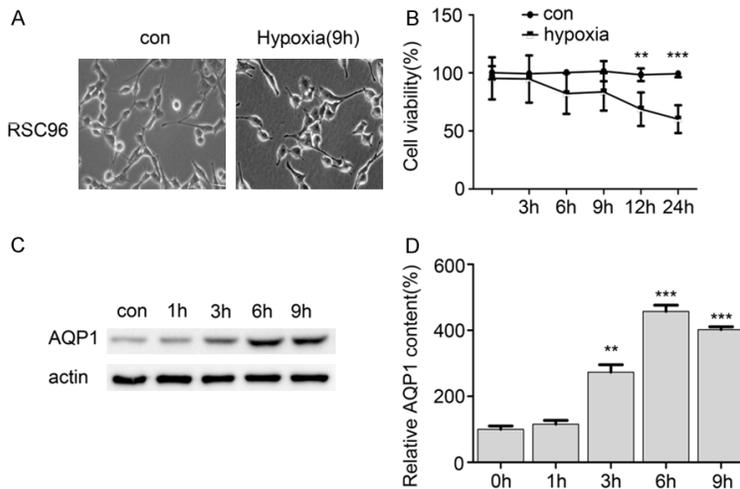

Figure 2. A. Hypoxia induces Schwann cells swelling. Phase contrast microscopy photographs of control group and hypoxia treated group after 9 h. B. The cell viability of hypoxia treated group significantly decreased compared to the control group with exposure time after 12 h (P < 0.01). C. The bands of AQP1 protein in Schwann cells line after hypoxia treated with western blot. D. Western blot analysis of AQP1 protein after hypoxia treatment. Levels were quantified from three independent experiments (n=3). Values are presented as means ± SD (n=3). (**P < 0.01, ***P < 0.001 compared to Control).

Results

Identification of AQP1 expression

The Schwann cells exhibited fusiform and long bipolar morphology (**Figure 1B**). To assess the cellular AQP1 expression, we used immunofluorescence and found that spindle-like cells were labeled (**Figure 1A**), the expression of AQP1 bands indicated that under normal circumstances the protein level was low in Schwann cells (**Figure 1C**). Therefore, we concluded that AQP1 was expressed in Schwann cells.

Hypoxia suppressed cell viability

Nine hours after induction of hypoxia, the cells began to swell. Most cells exhibited a round morphology compared to the control cells (**Figure 2A**).

Cell viability in the control group showed no significant changes between 0 and 24 h, while that in the hypoxia group significantly decreased with exposure time after 12 h (P < 0.01) (**Figure 2B**).

Hypoxia induced AQP1 expression at time dependent manner

When Schwann cells were induced with hypoxia at the indicated times (0 h, 1 h, 3 h, 6 h, and 9 h), the expression of AQP1 increased gradually after 3 h (P < 0.01) and peaked at 6 h (P < 0.001), as measured by Western blot analysis (**Figure 2C**, **2D**).

Hypoxia activated MAPK pathway in Schwann cells

After hypoxia was induced at different time points, the total amount of each MAPK in the cells remained constant. Hypoxia caused the activation of p-ERK, p-P38, and p-JNK in a time-dependent manner (**Figure 3A**). p-ERK was activated after 15 min (P < 0.01); the activation persisted and reached its peak at 1 h (P < 0.001, **Figure 3B**). p-p38 was activated after 15 min (P < 0.01) and peaked at 1 h (P < 0.001, **Figure 3D**). p-JNK was activated after 30 min (P
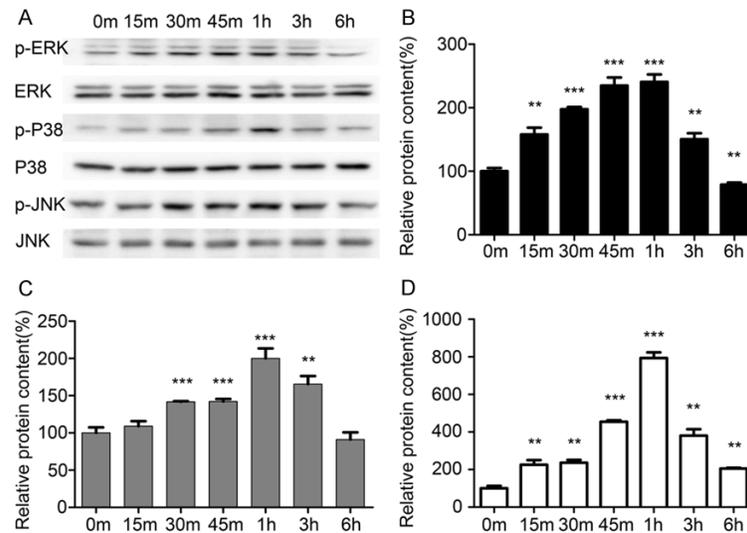
Figure 3. The effects of hypoxia on the activation of MAPK signaling pathway in Schwann cells. For the MAPK immunoblotting, cells were incubated in the Tris-gas (2% O_2) incubator for a series of times (A). cells were harvested. Immunoblot analysis was performed by using antibodies to p-ERK/ERK (B), p-JNK/JNK (C), p-p38/p38 (D), respectively. Representative blots and summary data are shown. Data is expressed as means ± SD (n=3). (**P < 0.01, ***P < 0.001 compared to 0 m).

(ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, are important intracellular signal transduction pathways. MAPKs have also been shown to be involved in the expression and regulation of AQP1 [19]. Previous studies have reported that hypoxia activated all three MAPKs and regulated AQP1 through other mechanisms as well [19]. Through an in vitro hypoxia model, we found that all three MAPKs, p-ERK, p-P38, and p-JNK, were activated, which was partly consistent with the results of our previous study with a CoCl$_2$ edema model [18]. However, the other possible mechanisms for AQP1 up-regulation through other signal pathways cannot be ignored. Other research groups have reported the involvement of PKC in the activation of p38-MAPK under hypoxia [20]; however, the underlying mechanisms remain obscure. Furthermore, Umenishi [21] had reported that under hypertonic stress, all three MAPK signal transducers were indispensable in the regulation of AQP1 [22].

Thus, it seemed that the regulation of AQP1 was important for more than one reason. Although previous studies have shown that the up-regulation of AQP1 was associated with facial nerve edema, related mechanisms have not been reported. According to our investigation, at the cellular level, currently available models only deal with hypoxia, while situations under osmotic pressure remain unexplored. However, as hypoxia and cell metabolism occur in vivo, osmotic pressure is sure to fluctuate as well, which would further complicate the matter [17, 23].

In conclusion, the present results demonstrated that hypoxia-induced up-regulation of AQP1 expression was regulated by the ERK/p38 pathway. Inhibition of each MAPK pathway resulted in significant reduction in hypoxia-induced AQP1 expression, indicating that MAPK signaling pathways were indispensable for the induction of AQP1 expression.

< 0.001) and peaked at 1 h (P < 0.001, **Figure 3C**).

MAPK inhibitor suppressed AQP1 up-regulation induced by hypoxia

In order to validate the role of the MAPK-dependent pathway, we investigated whether U0126, SB203520 and SP600125 (10 μM), which are specific inhibitors of the ERK, p-38 and JNK signaling pathways, respectively, could effectively prevent AQP1 up-regulation after hypoxia treatment. After 6 h of the treatment, the cell swelling under pretreated by U0126 (**Figure 4A**, **4B**) or SB203520 (**Figure 4C**, **4D**), was indeed inhibited. However, SP600125 (**Figure 4E**, **4F**) could not inhibit AQP1 expression.

Discussion

In this study, we demonstrated that hypoxia-induced swelling of Schwann cells caused a time-dependent up-regulation of AQP1. Pretreating the culture medium with U0126 and SB203580, specific inhibitors of ERK and p38, respectively, prevented this hypoxia-induced up-regulation of AQP1.

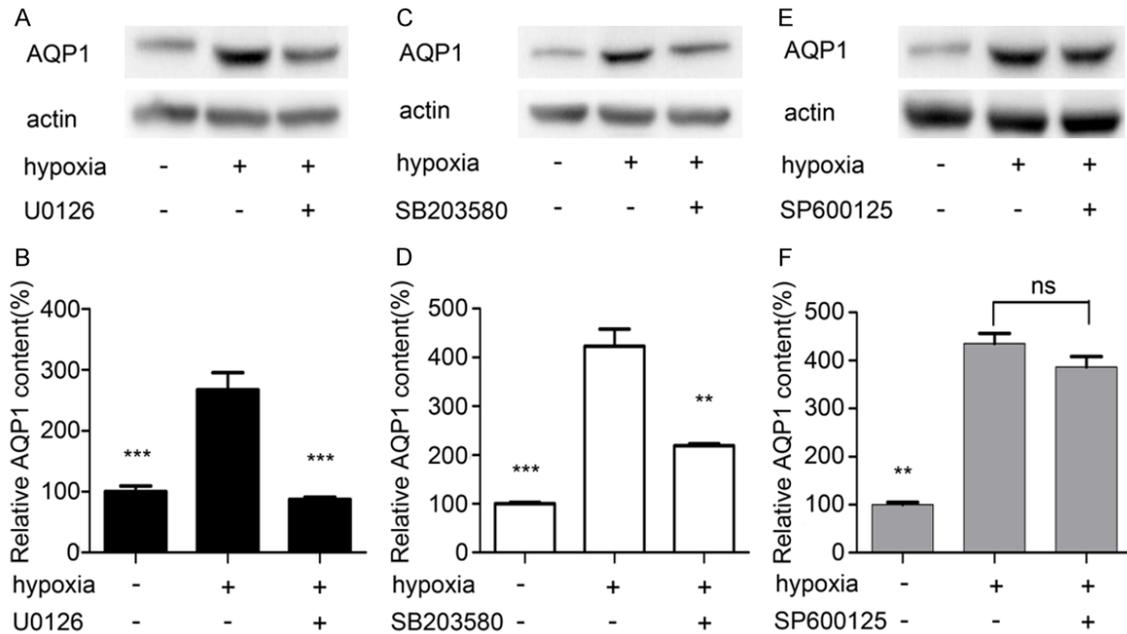Mitogen-activated protein kinases (MAPKs), especially extracellular signal-regulated kinase

Figure 4. The effect of hypoxia on AQP1 expression in the presence of ERK inhibitor U0126 (A, B), p-p38 inhibitor SB203580 (C, D), JNK inhibitor SP600125 (E, F). After pretreated with inhibitor (10 μM) or vehicle for 1 h, respectively, cells were treated with hypoxia for 6 h. Values are as means ± SD (n=3). (**P < 0.01, ***P < 0.001 compared to Hypoxia group).

Disclosure of conflict of interest

None.

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